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(54) Title: HIGH SPECIFICITY PRIMERS, AMPLIFICATION METHODS AND KITS

(57) **Abstract:** For nucleic acid amplification including extension of primers by a DNA polymerase, high specificity primers are provided. The primers include a type of hairpin structure in which a single-stranded loop separates complementary 3' and 5' arms and in which the loop and the 3' arm are complementary to the target nucleic acid. Amplification methods, assays and kits including such primers are included in the invention.

High Specificity Primers, Amplification Methods and Kits

This invention relates to nucleic acid detection that includes amplification of target sequences.

Background of the Invention

5   Amplification utilizing DNA primers and a DNA polymerase is a well known technique for detecting nucleic acid target sequences. Methods for exponential amplification include the polymerase chain reaction (PCR), strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), and rolling-circle

10   amplification (RCA). Among numerous DNA polymerases commonly used are Thermus aquaticus DNA polymerase and reverse transcriptase. The design of linear DNA oligonucleotide amplification primers is generally accomplished with the acid of a computer program designed for that purpose. Among the available programs that can be utilized are PRIDE (Haas et al. 1998), OLIGO (Rychlik et al. 1989), OSP

15   (Hilber et al. 1991), Primo (Li et al. 1997) and Primer Master (Proutski et al. 1996).

A common problem is known as "primer-dimers". Primer-dimers are false amplification products (amplicons) that are generated because two primers hybridize to each other with overhangs, thereby providing binding sites for a polymerase and initiating DNA synthesis. Primer-dimers compete with the intended amplification and

20   generally reduce the reliability and sensitivity of an assay. Another common problem is mis-priming of a sequence in a sample that is partially complementary to the primer. This also leads to false amplicons and reduces reliability and sensitivity.

One major application for target-amplification methods is _in vitro_ diagnostics. In diagnosing pathological conditions by nucleic acid-based techniques,

25   a common situation is that a unique nucleic acid sequence from a pathogen is a rare component of the total nucleic acid in a clinical sample. For example, the genomic DNA of the malarial parasite is a very small fraction of the total DNA that is extracted from a patient's blood. Amplification of rare pathogenic target sequences is an effective means for detection in some cases, because primers can be designed that

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successfully ignore the abundant human sequences sufficiently for diagnostic

purposes. However, there are many situations in which a rare target sequence is very

similar to an abundant sequence, differing in some cases by only a single nucleotide.

For example, certain human cancers are characterized by an alteration at just one

5   nucleotide position in a gene (Lengauer et al., 1998). To detect these cancers at an

early stage, or to detect their remnants after surgical removal of a tumor, it is

necessary to detect the presence of a rare sequence that differs from an abundant

sequence by only a single nucleotide. When a sequence that indicates the presence of

cancer is rare, the difficulty of detecting that sequence is sometimes referred to as the

10   "minimal residual disease problem." A similar problem arises when the emergence of

a drug-resistant bacterium or virus needs to be detected as early as possible when a

patient is being treated with a drug, because a number of drug-resistance genotypes

are characterized by a single nucleotide substitution in a pathogenic sequence. For

applications such as those described above, simple target amplification is not

15   effective, because the primers cannot sufficiently distinguish between two sequences

that differ from each other by only a single nucleotide substitution.

        Two approaches have been used to address this problem. The first is to

design one of the two oligonucleotide primers that are needed for amplification to

bind to the target at a sequence that encompasses the site of the nucleotide

20   substitution. If the primer is perfectly complementary to its intended target sequence,

then a primer-target hybrid will form, leading to the generation of amplified copies of

the target nucleic acid sequence. The hope is that if a nucleotide substitution is

present, then the mismatched primer-target hybrid will not form, resulting in an

inability to generate amplified copies of the nucleic acid sequence. However, this

25   dichotomy does not work well in practice, and both the mutant and the wild-type

templates result in amplification. The products of amplification of perfect and

mismatched targets (the "amplicons") are indistinguishable from one another. Even if

only mismatched target sequences are present in the sample, the primer will

occasionally initiate DNA synthesis on the mismatched target sequence. Because the

30   resulting product contains a perfect complement of the primer sequence, exponential

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amplification of this initial product occurs at a rapid rate. The second approach that is
used to detect mutations in a target sequence is to utilize primers that bind outside the
sequence that might contain a mutation, so that the sequence that contains the site of
the mutation becomes a part of the resulting amplicons. Additional hybridization
5   probes are then used to determine if the mutation is present within the amplicons. The
proportion of amplicons containing a mutation is a measure of the relative amount or
absolute amount of the mutation in the starting sample. Although this approach works
well in many situations (Tyagi et al. 1996, Tyagi et al. 1998), it has a sensitivity
limitation: if the mutant amplicons are less than a few percent of all the amplicons,
10 they cannot be detected.

       In order to detect mutants that are rare, that is, less abundant than the few
percent of the wild-type sequence that is needed for detection by hybridization probes,
an "amplification refractory mutation system" (ARMS) has been used (Newton et al.,
1989; Wu et al., 1989). In this method, two amplification reactions are carried out in
15 separate reaction tubes. The difference between the reactions is that one of the
primers is slightly different in each tube. The difference between the primers is in the
identity of the nucleotide at their 3' ends. The 3' nucleotide of the primer in one
reaction tube is complementary to the wild-type nucleotide at the site of mutation,
while the 3' nucleotide of the primer in the other reaction tube is complementary to
20 the mutant nucleotide at the site of mutation. If the primer in the tube is perfectly
complementary to its target sequence, including the nucleotide at the 3'-end of the
primer, then the primer can be efficiently extended by incubation with DNA
polymerase. However, if the binding of the primer in the tube to the target sequence
creates a mismatched 3'-terminal nucleotide, then the primer cannot be efficiently
25 extended by incubation with DNA polymerase. Amplification of the mismatched
template is significantly delayed, i.e., the number of thermal cycles in a polymerase
chain reaction (PCR) amplification that are required before the amplification product
can be detected (or the amount of time it takes to generate a detectable quantity of
amplification product in an isothermal amplification) is significantly greater when the

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3' nucleotide of the primer is not complementary to the sequence present in the sample.

ARMS primers and conventional primers are both prone to generating false-positive signals, because they can initiate the exponential synthesis of
5   unintended amplicons, even in absence of perfectly complementary target sequences. These "false amplicons" arise because the 3' end regions of the primers can bind to partially complementary sequences unrelated to the target that are present in samples. They also can arise from the binding of one primer molecule to another primer molecule, which results in the initiation of DNA synthesis (primer-dimers). In either
10   case, the resulting extension products can be exponentially amplified in the normal manner, resulting in the synthesis of false amplicons. The generation of false amplicons not only makes it difficult to identify the intended amplicons, but also limits the sensitivity of assays, since false amplicons compete with the intended amplicons, and thereby reduce their abundance. For example, if a rare target
15   sequence requires 38 cycles of PCR to be detectable above background, but a false amplicon in the reaction becomes detectable after 35 cycles, the rare target will not reach a detectable level.

The present invention markedly improves the specificity of oligonucleotide primers.

20   One aspect of the invention is an improvement in the sensitivity of assays that detect target nucleic acids that contain a single nucleotide substitution within a population of much more abundant wild-type nucleic acids, enabling detection at levels below a few percent.

Another aspect of the invention is a reduction in the formation of false
25   amplification products, including primer-dimers.

Another aspect of the invention is that it enables the determination of the fraction of a nucleic acid population that is mutant and the fraction that is wild type, particularly when the fraction is very small or very large.

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Another aspect of the invention is the decrease or elimination of the tendency of target amplification reactions to produce false amplicons.

Another aspect of the invention is to provide a means of labeling the amplification product with a fluorescent moiety so that the reactions can be monitored
5   in real time without having to utilize probes or nonspecific intercalating reagents.

Summary of the Invention

This invention includes oligonucleotide primers for nucleic acid amplification. When not bound to target, primers according to this invention form a particular type of hairpin structure in which the 3' terminal region of the primer is
10   hybridized to the 5' terminal region of the primer to form a double-stranded stem. Only the central region of the primer is single stranded and available for initial hybridization to a complementary target, a process sometimes referred to as "nucleation". This invention also includes amplification methods and assays that utilize such primers, and kits for performing such assays. These methods and assays
15   reduce false amplicon synthesis that limit existing methods and assays.

Certain primers according to this invention are useful to detect the presence of a mutant having a single nucleotide substitution in a generally wild-type population even when the amount of the mutant is below the detection limit, generally a few percent, currently achievable by the use of labeled detector probes or by the use
20   linear primers whose 3' terminal nucleotide hybridizes at the nucleotide subject to mutation. Having the loop of primers according to this invention hybridize to a target at a sequence containing the nucleotide subject to mutation permits detection of very low levels of mutant strands.

Amplification reactions and assays according to this invention utilize at
25   least one hairpin primer according to the invention. Exponential amplification reactions and assays (for example, the polymerase chain reaction) utilize a pair of primers, sometimes referred to as "forward" and "reverse" primers, one of which is complementary to a nucleic acid strand and the other of which is complementary to

the complement of that strand. Where a pair of amplification primers is used, either one or both are hairpin primers according to this invention.

Assays according to this invention may utilize any detection method for detecting amplicons. Such methods include gel electrophoresis, intercalating dyes,
5   minor groove binding dyes, fluorescence polarization, mass spectrometry and labeled detection probes. Detection may be end point, that is, carried out when amplification is completed, or real time, that is, carried out during the amplification process. Real-time probe-based detection methods include 5' nuclease assays (Gelfand et al., 1996; Livak et al., 1996) and molecular beacon assays (Tyagi et al., 1996; Tyagi et al.,
10   1997; Tyagi et al., 1998). Alternatively, primers according to this invention can be labeled with interactive fluorescent label pairs such as two fluorophores or a fluorophore and a non-fluorescent quencher, such that a change in fluorescence signal indicates the presence of primers that have been extended and, thus, the presence of a target for the primers in a sample. Interaction between labels may be by fluorescence
15   resonance energy transfer (FRET), by touching, or both. Assays utilizing labeled primers according to this invention can be real-time assays as well as end-point assays.

Kits according to this invention include amplification reagents, generally at least amplification buffer and dNTPs, and normally DNA polymerase, and at least
20   one primer according to this invention. Kits may include additional reagents, for example detection reagents, and may include instructions for performing an assay.

<u>Brief Description of the Drawings</u>

Fig. 1 is a schematic representation of the manner in which hairpin primers according to the invention are used to detect a single nucleotide substitution.

25   Fig. 2 shows the changes in fluorescence intensity of polymerase chain reactions (PCR's) according to this invention that detect a single nucleotide substitution as a function of the number of thermal cycles completed.

Fig. 3 shows how the PCR threshold cycle changes, when primers according to this invention are used, either mutant-specific primers or

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wild-type-specific primers, as a function of the proportion of mutant targets that are present in a mixture of molecules that contains both mutant and wild-type sequences (where the mutant sequence differs from the wild-type sequence by only a single nucleotide substitution).

5   Fig. 4 shows a standard curve that enables the threshold cycles measured in two different polymerase chain reactions (one reaction containing a wild-type-specific primer according to this invention and the other reaction containing a mutant-specific primer according to this invention) to be used to measure the relative abundance of mutant targets in a population of molecules that contains both

10   mutant and wild-type sequences (where the mutant sequence differs from the wild-type sequence by only a single nucleotide substitution).

Fig. 5 shows the nucleotide sequence and the mode of attachment of the fluorophore and the quencher moieties in a labeled primer according to this invention.

Fig. 6 shows the change in the fluorescence intensity of polymerase chain

15   reactions that utilize labeled primers according to this invention as a function of the number of thermal cycles completed.

Fig. 7 shows changes in the fluorescence intensity of polymerase chain reactions that utilize primers according to this invention as a function of the number of thermal cycles completed.

20   Detailed Description of the Invention

An important aspect of this invention is target-amplification methods and assays that are less prone to generating false amplicons and that are more sensitive than amplification methods and assays currently in use involving amplification of rare sequences, including the detection of rare mutant sequences within a population of

25   wild-type sequences, or vice versa. Assays based on this invention use primers that can exist as hairpins comprising a single-stranded "loop" and a double-stranded "stem". With reference to FIG. 1, these primers 1 consist of three parts, a 5'-arm sequence 2, a central loop sequence 3, and a 3'-arm sequence 4 that is complementary to 5'-arm sequence 2. The loop 3 and the 3' arm 4 of the hairpin 1 are complementary

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to one strand of target 5, whereas the 5' arm 2 is designed to convert the primer into a hairpin-shaped molecule. Thus, primers according to this invention have both loops and 3' arms that are complementary to the target and a 5' arm that competes with the target for hybridization to the 3' arm. When the sequence in the loop 3 binds to its

5   complement in the target 5, the stem of the hairpin-shaped primer 1 unravels and the 3' arm 4 anneals to the target. This creates the substrate for enzymatic extension of primer 1. Target 5 may be a single strand, or it may be double-stranded. In either case second primer 6, which may be a conventional linear primer, permits exponential amplification to produce double-stranded amplicon 7. Primers according to this

10   invention have stems that do not unravel, or dissociate, unless both the loop and the 3' arm are complementary to the target. If a primer according to this invention is tested against a model non-target of the length of the loop and perfectly complementary to the loop the stem does not dissociate.

       There are special cases in which at least a portion of 5' arm is at least

15   partially complementary to the intended target. Following the design criteria discussed below, one may design a primer which has one or a few 3'-terminal nucleotides of the 5' arm complementary to the target. Example 4 below illustrates this situation. Instances in which the entire 5' arm will be complementary to a target will be rare.

20   Because the 3' portion of the primer is in a double-stranded state, the generation of false amplicons by primer-dimer formation or mis-priming of 3' ends is prohibited to a very large extent. If the sample contains a nucleic acid differing from target 5 by a nucleotide that is not complementary to the sequence in loop 3, the loop cannot bind to that nucleic acid and 3' arm 4 cannot anneal to the nucleic acid and

25   initiate DNA synthesis. Consequently, the presence of target molecules (for example, either wild-type or mutant DNA template molecules, or wild-type or mutant RNA template molecules) in the sample results in amplicon synthesis, whereas the presence of molecules differing from the target sequence by as little as a single nucleotide substitution either does not result in amplicon synthesis, or if the primer happens on

very rare occasions to bind to the mismatched template and initiate amplicon synthesis, the synthesis of the amplicons will be significantly delayed.

Referring to FIG. 1, it will be seen that in the embodiment depicted there, the terminal nucleotides of the primer are complementary and form part of stem. We

5   refer to them as "arms". This is also true for the embodiment shown in FIG. 5. This is our preferred construction utilized in the several examples described below. However, it is within the scope of the invention that non-complementary nucleotides be included within the stem, which will reduce the strength of the stem, or included as non-complementary terminal nucleotides or the stem, for example, a single-stranded

10   overhang. The latter presents a danger of false priming, so any overhang must be incapable of nucleation with other nucleic acid sequences so as to provide a starting point for branch migration. In primers according to this invention nucleation is by annealing of the loop sequence to the target. It will be understood that as used in this application and the appended claims "stem" and "complementary 3' and 5' arm

15   sequences" are sufficiently broad to encompass the above variants.

It will be appreciated that, even when the 5' arm is completely non-complementary to the intended target, amplicons synthesized by copying oligonucleotides containing a primer will contain complete complements of the primer. This may reduce the discriminatory effectiveness, including the suppression

20   of false amplicons, achieved in the first round of synthesis. To preserve to initial effect throughout amplification, certain preferred embodiments of primers according to this invention include a "terminator nucleotide" inserted between the loop and the 5' arm. As used herein, "terminator nucleotide" is a nucleotide that stops extension by a polymerase during DNA synthesis. The terminator nucleotide may be, for example,

25   4-methylindole β-deoxynucleoside (Moran et al., 1996). Insertion of a terminator nucleotide between the 5' arm and the loop prevents copying of the 5' arm.

Hairpin primers of the present invention can contain deoxyribonucleotides, ribonucleotides, peptide nucleic acids (PNA), other modified nucleotides, or combinations of these. Modified nucleotides may include, for example,

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2'-O-methylribonucleotides or nitropyrole-based nucleotides. Modified
internucleotide linkages may also be included, for example phosphorothioates. Using
modified nucleotides in the 5' arm provides a way to adjust the strength of the stem.
Other advantages of using such modifications for a particular application will be
5   apparent to persons familiar with the art. In particular, hairpin primers according to
this invention that are constructed from modified nucleotides may form stronger
hybrids than if the primers were constructed from deoxyribonucleotides, thus enabling
structured target sequences (such as those that occur in messenger RNAs) to be more
easily accessed.

10   Certain embodiments of primers according to this invention are highly
discriminatory against single nucleotide substitutions. In these embodiments the loop
is complementary to the target region containing the target nucleotide subject to
substitution. We place that nucleotide near the center of the loop. In addition we
attempt to make the loop as short as possible, that is, from five to nine nucleotides
15   long, to maximize the impact of a single mismatch.

            Hairpin primers of the present invention permit monitoring of
amplification reactions by fluorescence. They can be labeled with interactive
fluorescent moieties, and with other labels, in such a manner that free primers permit
the interaction of the label moieties, whereas the labels of primers that are
20   incorporated into double-stranded amplicons do not so interact. One of the
interactions that are useful in detection is fluorescence energy transfer, which causes
the fluorescence of free primers to be quenched and the fluorescence of those that are
incorporated into amplicons not to be quenched. As a result, the course of the
amplification reactions can be monitored by measuring the fluorescence. Particularly
25   useful is the interaction of quenching by touching, wherein a non-fluorescent
quenching molecule such as DABCYL, DABMI, DABSYL or Methyl Red is used to
prevent a fluorophore from fluorescing, rendering free-floating primers dark. Because
(a) hairpin primers according to this invention are so specific that they are extremely
unlikely to generate false amplicons, and (b) their particular hairpin shape markedly
30   reduces the probability that they will interact with each other to form primer-dimers,

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the generation of a fluorescent signal in a reaction that utilizes hairpin primers according to this invention is an excellent indication that the intended amplicons have been synthesized. Furthermore, the generation of a fluorescent signal during real-time detection of the amplification products allows accurate quantitation of the initial

5   number of target sequences in a sample. This represents an improvement over the current art. In the current art, either hybridization probes have to be used to monitor the amplification reactions, or when other methods of amplicon labeling are used, these methods are not sufficiently specific to discriminate false amplicons from the intended amplicons (Nazarenko et al., 1997; Wittwer et al., 1997).

10   The advantages of using hairpin primers according to this invention are unexpected. It has been a common belief that the presence of a hairpin in the priming region of a primer, deleteriously diminishes the ability of the primer to prime. Examples of such teachings can be found in computer programs that are used to design primers for polymerase chain reactions, where the algorithms reject putative

15   primer sequences that possess self-complementary (hairpin-forming) sequences (for example, Haas et al., 1998; and Rychlik et al., 1989).

Hairpin primers of the present invention can be used to solve the problem of false amplicon synthesis in amplification reactions. Conventional primers, that is, primers whose target-complementary regions are linear, are prone to producing false

20   amplicons because their 3' ends can bind to partially complementary sequences in either unrelated DNA or in other primers, where they initiate DNA synthesis, generating unintended templates for DNA polymerase. These templates are then amplified normally, resulting in false-positive signals. However, the sequence at the 3' end of a hairpin primer according to this invention is hybridized to the sequence at

25   the 5' end, and thus almost never binds to a target sequence unless the loop sequence first anneals to the target sequence. The hybrid formed by the binding of the loop sequence to a portion of the target sequence then lengthens by branch migration, resulting in the binding of the 3□-arm sequence to the remainder of the target sequence. This process can not occur at all or only very rarely unless the target

30   sequence is perfectly complementary to the loop sequence. Partially complementary

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sequences almost never take part in this chain of events, and priming does not occur. Therefore, except on very rare occasions, priming occurs only at perfectly complementary sites on target strands.

Hairpin primers of this invention are useful in a number of nucleic acid
5 amplification processes that employ primers, including polymerase chain reactions (PCR), strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), and rolling-circle amplification (RCA). The high specificity and consequent low mis-priming significantly and detectably delays signal from sequences other than perfectly
10 complementary targets. In thermal cycling amplifications, this delay is manifested in a later threshold cycle: in isothermal amplifications, in a later time for detectable signal to arise.

Nucleic acid amplification assays that utilize hairpin primers of the present invention can be designed so that the amplicons are detected by conventional methods
15 at the end of the reaction, such as by polyacrylamide gel electrophoresis, or the products can be detected in real time during amplification using conventional detection methods. A variety of real-time detection methods, including the use of intercalating dyes and probe-based methods, can be employed (reviewed by Zubritsky, 1999), or the fluorescence of labeled hairpin primers themselves can be
20 utilized, as described in Example 3 below.

In order to determine the fraction of mutant and wild-type sequences in a sample that are mutant, a portion of the DNA in the sample is amplified in one reaction using a wild-type-specific primer according to this invention, and a second portion of the DNA in the sample is amplified in a second reaction using a
25 mutant-specific primer according to this invention. If amplification includes thermal cycling, the difference in the threshold cycles of the two reactions indicates the relative proportion of the mutant and the wild-type sequences in the original sample. The larger the difference, the smaller is the fraction of mutants. If amplification is

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isothermal, the difference in the time required for detectable signal to arise indicates the relative proportion.

Hairpin primers according to this invention can be prepared by first designing the priming sequence, the portion complementary to the intended target, that is, loop 3 and 3' arm 4 (FIG. 1), in the conventional manner. For this purpose numerous design programs are available and have been reported in the literature. See, for example, Haas et al. (1998), Rychlik et al. (1989), Hillier et al. (1991), Li et al. (1997) and Proutski et al. (1996). Generally, these programs provide a primer sequence 15-20 nucleotides long, slightly longer on occasion. Next, 5' arm 2 (FIG. 1) is designed as an oligonucleotide sequence complementary to 3' arm 4 (FIG. 1) so as to leave 5-9 nucleotides for the loop 3 (5 nucleotides for short priming sequences and 9 nucleotides for priming sequence of 19 or more nucleotides). However, if discrimination against single nucleote changes is not required, the length of the loop can be increased by several nucleotides (see Example 4), the primer with a loop thirteen nucleotides long.

In designing primers according to this invention that can initiate DNA synthesis from a perfectly complementary DNA sequence but not from a sequence that contains a single nucleotide substitution, it is necessary to maximize the specificity of the primer. Generally, the smaller the primer the more specific priming is. However, primers that are smaller than a certain length will not bind to the complementary sequence, and thus will not be able to prime DNA synthesis. Hairpin primers of the present invention achieve high specificity by virtue of their stem. The sequence of the 3' arm of the hairpin stem and the sequence of the hairpin loop are complementary to the intended (perfectly complementary) target sequence, whereas the 5'-arm sequence of the hairpin stem does not bind to the intended target sequence. However, amplicons that are generated by copying template molecules that are extension products of hairpin primers do contain a perfect complement of the entire hairpin primer sequence, and thus the 5'-arm sequence of the hairpin primer, as well as the loop sequence and 3□-arm sequence, will bind to these amplicons in subsequent rounds of amplification. The early priming events are therefore critical in

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determining the specificity of amplification. The longer (or stronger) the stem of the hairpin primer is, the more specific its annealing with the template will be, and therefore the more specific the priming reaction will be. However, making the stem longer also increases the total length of the primer, which makes the primer less

5   specific. The independent optimization of each of these two design criteria would have an opposite effect on the length of the stem. There is thus an optimal length for the stem at which maximum discrimination can be achieved. Since the stem sequence is determined by the sequence of the target around the site of mutation, the optimal length will depend on the identity of the target sequence. The optimum length of the

10   stem usually lies between 5 to 12 deoxyribonucleotides, which is our preferred range. The lower limit is for GC-rich sequences and the upper limit is for AT-rich sequences. DNA folding programs can be used to design the hairpin structures of the primers and to predict their stability (Serra and Turner, 1995).

        The initial contact between the hairpin primer and the target sequence

15   is made by the loop of the hairpin primer, because the 3' arm is bound to the 5□ arm, and is therefore unavailable for initial hybridization to the target sequence. If the target is recognized to be perfectly complementary, the 3' arm dissociates from the 5' arm and binds to the target. However, if the target sequence is not perfectly complementary to the loop sequence, then the initial contact is short-lived and the

20   primer dissociates from the target sequence. The capacity to discriminate between a perfectly complementary target sequence and a mismatched target sequence increases as the length of the loop is decreased. However, very short loops will be too small to anneal to the target. Loops of 5 to 9 deoxyribonucleotides are optimal.

        A primer initially designed as described above can be tested in the

25   intended assay utilizing perfectly complementary target molecules and in the intended assay utilizing molecules that differ by a single nucleotide. Such a test is described below in Example 1. The purpose of the test is to determine whether the degree of specificity required for the assay has been achieved. A primer according to this invention is at least 1000 times as efficient in priming the complementary target as it

30   is in priming the sequence with the nucleotide mismatch, preferably 5000 times as

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efficient. We have made primers according to this invention that are 10,000 times as efficient without extensive optimization. If the primer as initially designed does not have sufficient specificity in the amplification method or assay being used, the strength of the stem should be increased. This can be, for example, by increasing the

5   length of the 5' arm sequence by adding a nucleotide (which maintains the length of the priming sequence). An alternative to changing the length is to substitute modified nucleotides in the 5' arm. Another alternative, and one which is a preferred embodiment, is to add a terminator nucleotide between the loop and the 5' arm. The terminator nucleotide may be, for example, 4-methylindole β-deoxynucleoside

10   (Moran et al., 1996). By using a terminator nucleotide, generated amplicons do not include the sequence complementary to the primer's 5' arm. This maintains the initial specificity in rounds of synthesis subsequent to the first round, or cycle, and thereby increases overall specificity. If the primer fails to result in amplification of its perfectly complementary target, the length of the loop should be increased. Persons

15   skilled in the art will be able to readily optimize the primer in the manner described.

The temperature of the annealing stage during amplification is a critical parameter that influences the specificity of priming. In polymerase chain reactions, this temperature can be adjusted in order to maximize specificity. This adjustment cannot be made with methods of amplification that are isothermal. The

20   higher the annealing temperature, the greater is the specificity. This is also true for hairpin primers of the present invention. The combined length of the loop sequence and the 3□-arm sequence is chosen in such a way that the initial probe-target hybrid that is formed by the annealing of the hairpin primer to a target sequence that is present in the sample will dissociate at a temperature that is slightly above the

25   annealing temperature of the amplification reaction. The precise annealing temperature that provides maximum discrimination can be found empirically by either systematically changing the annealing temperature and determining the degree of discrimination, or by carrying out these measurements with a gradient thermal cycler that can employ a range of temperatures for the annealing stage.

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Primers of the present invention can be labeled with interactive label
moieties so that their incorporation into the products of amplification can be
monitored by fluorescence. The fluorescence of primers that are incorporated into
amplicons is very different from those that are not incorporated. This permits

5   identification of the products of amplification without any further analysis.
Furthermore, the use of interactive label moieties allows accurate quantification of the
initial number of target molecules by performing real-time detection of the amplicons
as they are synthesized.

We have described optimal methods of selecting and attaching

10   interactive fluorophores and quenchers (as well as other label moieties) to
hairpin-shaped probes called "molecular beacons" (Tyagi and Kramer, 1996; Tyagi et
al., 1997; Tyagi et al., 1998). In order to obtain higher signal-to-background ratios in
assays that use molecular beacons, it is desirable to have the fluorophore and the
quencher be very close to each other in the hairpin state of these probes and far from

15   each other in their target-bound state. A similar mode of attachment of the two labels
is ideal for hairpin primers according to this invention. However, in molecular
beacons the quencher moiety DABCYL is usually attached to the 3'-hydroxyl group,
which serves to block extension of the probe at its 3' end when DNA polymerase is
present. However, in a hairpin primer (whose purpose is to initiate the synthesis of a

20   copy of the template strand) an unblocked 3'-hydroxyl group is required for extension
to occur. This precludes the attachment of a label to the 3'-hydroxyl position. We
have explored other ways of attaching the labels. In a first method, the fluorophore is
attached to the 5' end of the 5' arm of the primer, as is the case in molecular beacons;
however. the quencher is attached to an internal location within the 3' arm. The

25   location of the quencher within the arm that brings the fluorophore and the quencher
close to each other is about 4 to 6 nucleotides away from the 3' end. In a preferred
embodiment. the quencher is attached to a thymidine nucleotide at one of these
positions (if the sequence allows it). The attachment is accomplished by using a
thymidine derivative that possesses a 6-carbon spacer emanating from the thymidine

30   ring and which terminates in a primary amino group. The quencher can be conjugated

16

to this primary amino group, using methods described by Tyagi and Kramer (1996).
Alternatively, a thymidine phosphoramidite that contains the spacer and the quencher
can be used during automated DNA synthesis, obviating the need for post-synthetic
steps. Example 3 describes one such primer. In a second method thymidine residue
5   that bears the quencher can also be placed at the 3' end of the primer. The resulting
molecule serves as a good primer because the 3'-hydroxyl moiety is available for
extension and is quenched very well (the two label moieties are close to each other
and they are free to interact with each other).

            These primers can be labeled with a variety of fluorophores that
10   fluoresce in different color ranges. A number of these differently colored hairpin
primers can be used in the same reaction mixture, so that copies of different target
sequences can be generated simultaneously, and each type of amplicon will fluoresce
in its own characteristic color (determined by the identity of the fluorophore linked to
its hairpin primer. Methods described by Tyagi, et al. (1998) can be used to
15   accomplish the labeling and appropriate quenching of the fluorophores in these
primers.

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Example 1

We designed a target-amplification assay for the detection of a single nucleotide substitution, a point mutation, in the gene for human methylenetetrahydrofolate reductase, whose presence within the gene increases the

5   risk of cardiovascular disease (the mutation is called the "C677T mutation"). We selected the polymerase chain reaction (PCR) as the amplification process. A pair of forward hairpin primers was designed to recognize the sequence of the gene within which the mutation is located. Each of these primers consisted of three parts, a 5' arm, a central loop, and a 3' arm that is complementary to the 5' arm. The sequence

10   formed by the 3' arm and the loop was complementary to the target sequence (which encompassed the site of the C677T mutation). The two hairpin primers were identical, except for one nucleotide in the loop. One of the primers was complementary to the wild-type target sequence and the other primer was complementary to the mutant target sequence. The sequence of the wild-type-specific

15   primer was 5'-<u>GATGAAAG</u>GAGCCGA<u>TTTCATC</u>-3' and the sequence of the mutant□specific primer was 5'-<u>GATGAAAG</u>GAGTCGA<u>TTTCATC</u>-3', where underlines identify the arm sequences and a bold letter in each sequence identifies the nucleotide that is perfectly complementary to one of the variant nucleotides that can occur at the site of the C677T mutation. Each of these forward primers was used in a

20   separate reaction in combination with a common reverse primer (a conventional linear primer) to amplify a small segment of the methylenetetrahydrofolate reductase gene. One of the two polymerase chain reactions was initiated with wild-type template, and the other polymerase chain reaction was initiated with mutant template, and the progress of each reaction was monitored in real-time with the aid of an intercalating

25   dye, SYBR Green, to render the resulting amplicons fluorescent. Also, as a control, we used a conventional forward primer in place of the hairpin primer, whose target sequence was outside the site of the C677T mutation. This "neutral" forward primer enabled the synthesis of wild-type amplicons and mutant amplicons at the same rate.

The conditions of the assays were such that each 50-□l reaction

30   contained 20,000 template DNA molecules (either mutant or wild-type), 0.1 □M of

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the wild-type-specific forward hairpin primer (or 0.5 ☐M of the conventional forward primer whose target sequence was outside of the site of the C677T mutation), 0.5 ☐M of conventional reverse primer, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.50 mM dUTP, 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer), 50

5   mM KCl, 3.5 mM MgCl₂, 1X SYBR Green (Molecular Probes, Inc.), and 10 mM Tris-HCl (pH 8.3). Forty cycles of amplification (94☐C denaturation for 30 sec, 55☐C annealing for 60 sec, and 72☐C polymerization for 30 sec) were carried out in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler in sealed tubes. Green fluorescence was monitored in real time during the annealing stage of

10 each cycle. Fluorescence as a function of PCR cycle number for each amplification is shown in FIG 2., where curve 21 is from the reaction containing the linear forward primer and 20,000 molecules of wild-type template; curve 22, the linear forward primer and 20,000 molecules of the mutant template; curve 23, the wild-type-specific hairpin primer and 20,000 molecules of wild-type template; and curve 24, the wild-

15 type-specific hairpin primer and 20,000 molecules of the mutant template.

The increase in the fluorescence of each of the four reactions that were performed is shown in FIG. 2. The "threshold cycle," which is the thermal cycle at which each reaction became positive (i.e., the thermal cycle at which the intensity of the fluorescence signal became 10 times as strong as the standard deviation of the

20 florescence background), is indicative of the number of template molecules that were initially present in the reaction. When the conventional, or neutral, primer was used as the forward primer (in place of one of the hairpin primers), the same threshold cycle (thermal cycle 16) was observed irrespective of whether 20,000 molecules of the wild-type template was initially present or whether 20,000 molecules of the

25 mutant template was initially present, indicating that the reactions were initiated with same number of template molecules (curves 21, 22). However, when the wild-type-specific hairpin primer was used, the reaction initiated with 20,000 molecules of wild-type template became positive after 20 cycles (curve 23), whereas the reaction initiated with 20,000 molecules of mutant template only became positive

30 after 31 cycles (curve 24). Since the number of amplicons doubles in every thermal

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cycle, the 11-cycle difference in the threshold cycle observed for each reaction indicates that the "delayed" reaction was initiated with 1/2,000th as many template molecules as was the "faster" reaction. However, these two reactions were actually initiated with the same number of template molecules. The apparent delay indicates

5   that the wild-type-specific primer was 1/2,000th as efficient in priming amplicon synthesis when mutant template was used to initiate the reaction than it was when wild-type template was used to initiate the reaction. These results imply that assays containing hairpin primers can detect mutant target molecules that are 1/2,000th as abundant as wild□type target molecules. In other experiments that utilized the same

10   templates and the same hairpin primers (and also in experiments that utilized different templates and appropriate wild-type-specific and mutant-specific hairpin primers) we have observed threshold cycle differences as great as 14 thermal cycles. These results demonstrate that assays containing hairpin primers can be used to detect mutant target molecules that are 1/10,000th as abundant as wild-type target molecules.

15   Example 2

        In order to demonstrate the utility of hairpin primers according to this invention for the detection of rare mutant targets in a population containing abundant wild-type targets, we performed a series of polymerase chain reactions that were initiated with mixtures of mutant templates and wild-type templates. The proportion

20   of mutant templates in these mixtures was varied from 0, 0.1, 1.0, 50.0, 99.0, 99.9, to 100 percent, with the total concentration of template DNA always being the same (20,000 molecules). Each of these mixtures was amplified using a wild-type-specific hairpin primer in one reaction and the corresponding mutant-specific hairpin primer in a second reaction. These primers differed by a single nucleotide. The conditions of

25   the reactions were the same as described for Example 1. The threshold cycles that were obtained with each template mixture are plotted in FIG. 3, in which curve 32 shows the results obtained for reactions that contained wild-type-specific primer, and curve 31 shows the results obtained for reactions that contained mutant-specific primer. The results show that pure wild-type templates give a low threshold cycle

30   with wild-type-specific primers and a high threshold cycle with mutant-specific

primers. The reverse is true for pure mutant templates. While the difference in the threshold cycles that are obtained is maximum for the pure templates, the difference decreases as the proportion of one type of template is raised and the proportion of the other type of template is decreased. As little as 0.1 percent of the mutant template in

5   a population that contained 99.9 percent the wild-type template is distinguishable from a pure wild-type template. FIG. 4 shows how the data obtained in the experiment shown in FIG. 3 can be used to establish a standard curve in which the difference between the threshold cycles (TC) measured in two different amplification reactions, here polymerase chain reactions, carried out with each sample (one reaction

10   containing the wild-type-specific hairpin primer and the other reaction containing the mutant-specific hairpin primer) is plotted against the fraction of mutant targets in the population of mutant and wild-type targets that are present in the sample. The difference is obtained by subtracting the threshold cycle obtained from the reaction containing the mutant-specific hairpin primer from the threshold cycle obtained from

15   the reaction containing the wild-type-specific hairpin primer. This curve 41, which was obtained using known samples, can be utilized as a standard to determine the proportion of mutant templates that are present in an unknown sample. These results show that hairpin primers of the present invention are suitable for diagnosing minimal residual disease that originates from single nucleotide substitutions.

20   Example 3

         In order to demonstrate that hairpin primers according to this invention can be labeled in such a way that their fluorescence increases upon their incorporation into amplicons, we synthesized a hairpin primer 51 whose sequence is shown in FIG. 5. The primer had a loop sequence eight nucleotides long, a stem seven nucleotides long,

25   resulting in a target-complementary sequence 15 nucleotides long. A DABCYL quencher moiety 52 was covalently linked to the fifth nucleotide from the 3' end of the oligonucleotide (which was a thymidine nucleotide) via a hexalkyl spacer 53 and a fluorescein moiety 54 was covalently linked to the 5' end of the oligonucleotide, also via a hexalkyl spacer 55. The methods described by Tyagi and Kramer (1996) were

30   utilized for the construction of this primer. We found that the fluorescence of the

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fluorophore 54 in this primer was quenched when the primer was incubated in the absence of target nucleic acids. When this primer was incubated in the presence of perfectly complementary targets, its fluorescence increased substantially. This primer was utilized in two polymerase chain reactions carried out under the same conditions

5   described for Example 1, with the exception that the intercalating fluorescent dye, SYBR Green, was omitted. The first reaction was initiated with 20,000 template molecules that contained a target sequence that was perfectly complementary to the loop sequence and 3' arm sequence of the hairpin primer, whereas the second reaction contained no template DNA. The intensity of fluorescein fluorescence was monitored

10   in real-time during each annealing stage of the polymerase chain reaction, and the results are plotted for both reactions as a function of the number of thermal cycles completed in FIG. 6. The results show that the fluorescence of the reaction mixture initiated with template DNA, curve 61, increases dramatically when the polymerase chain reaction enters the linear phase. However, the fluorescence of the reaction that

15   did not contain any template DNA, curve 62, remained low throughout the course of the polymerase chain reaction. A comparison of the fluorescence intensity of the two reaction mixtures at the end of the amplification process indicates that their fluorescence is so different that the reaction initiated in the absence of template DNA could easily be distinguished from the reaction initiated with template DNA simply by

20   illuminating the reaction tubes with an ultraviolet lamp. We also synthesized hairpin primers according to this invention that utilized a different labeling scheme from the one mentioned above, and we tested the resulting labeled hairpin primers. In this alternative labeling scheme, the 3' end of the primer was a thymidine nucleotide that was covalently linked to a fluorescein moiety via a hexalkyl spacer that was linked to

25   the nucleotide, rather than being linked to the 3'-hydroxyl group of the deoxyribose moiety. This primer contained a 5'-DABCYL moiety. The primer was well quenched and its fluorescence was restored when it was incorporated into amplicons. Despite the linkage of the fluorescein moiety to the 3' nucleotide, this primer was extended normally by incubation with DNA polymerase in an amplification reaction.

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Example 4

In order to demonstrate that hairpin primers of the present invention solve the problem of false amplicon synthesis during amplification reactions, we utilized a pair of conventional linear primers that normally produce false amplicons when the amplification reactions that employ them are initiated without any template DNA. Using the sequence of these primers, we designed and constructed hairpin primers according to this invention for the same target sequences as the conventional primers were designed to bind to. We then demonstrated that when the corresponding hairpin primers are used in place of the conventional primers in a polymerase chain reaction, they generate the expected amplicons in reactions initiated with target template DNA, but (unlike reactions containing conventional primers) they do not generate false amplicons in reactions initiated without template DNA. The sequences of the first conventional primer was 5'-GGCCGGTGGTCGCCGCG-3' and the sequence of the second conventional primer was 5'-ACGTGACAGACCGCCGGGC-3'. The sequence of the first corresponding hairpin primer was 5'-CGCGGCCGGTGGTCGCCGCG-3' and the sequence of the second corresponding hairpin primer was 5'-GCCCGGACGTGACAGACCGCCGGGC-3', where underlines identify the arms of the hairpins. These primers were designed to amplify a region of the RNA polymerase gene of *Mycobacterium tuberculosis*.

The design of the first and second hairpin primers is illustrative of two design points. For the first hairpin primer we noted that a loop eight nucleotides long and a stem six nucleotides long could be obtained by adding only three nucleotides to the 5' terminus of the first conventional primer. That was possible in this particular instance, because of fortuitous complementarily. Note that three nucleotides in the 5' arm are there nucleotides at the 5' end of the first conventional primer. For the second hairpin primer the 5' arm includes six added nucleotides. However, in this instance note that the loop, thirteen nucleotides long, is outside the preferred range of 5-12 nucleotides. In this instance we were not attempting to discriminate against a single nucleotide substitution. Four polymerase chain reactions were performed, two

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containing the conventional primers, and two containing the hairpin primers. One of the reactions in each pair was initiated with target template DNA, whereas the other reaction in the pair did not contain any template DNA. The progress of these reactions was monitored using the fluorescent intercalating dye, SYBR Green, to label

5   any amplicons that were generated in the course of the amplification reactions. The changes in fluorescence intensity that were observed during the course of the amplification reactions is shown in FIG. 7. The results show that the reaction that contained the first and second conventional primers and 20,000 template molecules, curve 71, became positive after 16 thermal cycles had been completed, and the

10   reaction that contained those conventional primers and no template DNA, curve 73, became positive after 29 thermal cycles had been completed, indicating that false amplicons were generated in the course of the reaction. A subsequent analysis of these amplicons by polyacrylamide gel electrophoresis showed that legitimate amplicons were produced in the first reaction, whereas false amplicons of an

15   unexpected size were produced in the second reaction. We believe the false amplicons were primer-dimers. However, when the first and second hairpin primers were used in place of the conventional primers, the reaction initiated with 20,000 target template molecules, curve 72, became positive after 17 thermal cycles had been completed, but the reaction that did not contain any template molecules, curve 74,

20   never became positive. Subsequent analysis of the amplicons by polyacrylamide gel electrophoresis confirmed that the first reaction generated the expected amplicons, and the second reaction did not generate any amplicons. These results confirm that hairpin primers of the present invention are useful in solving the problem of false amplicon synthesis during amplification reactions. The reason that hairpin primers

25   suppress the synthesis of false amplicons is that only the sequence in the loop is available for initiating the primer-template hybrid, and the presence of a hybridization sequence in a hairpin loop renders the interaction between the primer and the target nucleic acid much more specific than the interaction that occurs when a conventional linear primer hybridizes to a target nucleic acid. Moreover, the structure of hairpin

30   primers is such that the sequences that are present in the arms of the primer do not

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participate in the initial hybridization of the primer to the target nucleic acid. Consequently, the binding of one primer to another during an amplification reaction (which can create undesirable amplifiable primer-dimers when conventional linear primers are used) is much less likely to occur when hairpin primers are used.

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We Claim:

1. An hairpin oligonucleotide primer for extension by a DNA
polymerase comprising a stem formed by complementary 3' and 5' arm sequences and

5   a single-stranded loop sequence separating said arm sequences, wherein said 3' arm
sequence and said loop sequence are both perfectly complementary to a selected
priming region of a target nucleic acid strand and wherein hybridization of the loop
sequence to a model non-target sequence of the length of the loop and perfectly
complementary to the loop sequence does not cause dissociation of the stem.

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2. The hairpin primer according to claim 1 additionally comprising
interactive fluorescent label moieties attached to the 3' and 5' arm sequences, whereby
incorporation of the primer into a double-stranded nucleic acid detectably alters the
fluorescence emitted by said label moieties.

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3. The hairpin primer according to claim 2 wherein said interactive
fluorescent label moieties comprise a fluorophore and a non-fluorescent quencher.

4. The hairpin primer according to claim 1 wherein said loop sequence

20   is 5 to 9 nucleotides in length and said complementary 3' and 5' arm sequences form a
stem that is 5 to 12 nucleotides in length.

5. The hairpin primer according to claim 4 wherein said priming region
contains a single nucleotide subject to mutation and said loop sequence is

25   complementary to the portion of said priming region containing said nucleotide.

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6.   The hairpin primer according to claim 4 wherein said primer contains a terminator nucleotide between the loop sequence and the 5' arm sequence.

7. The hairpin primer according to claim 4 containing modified
5   nucleotides, modified internucleotide linkages, or both.

8.   The hairpin primer according to claim 4 additionally comprising interactive fluorescent label moieties attached to the 3' and 5' arm sequences, whereby incorporation of the primer into a double-stranded nucleic acid
10   detectably alters the fluorescence emitted by said label moieties.

9.   The hairpin primer according to claim 6 wherein said interactive fluorescent label moieties comprise a fluorophore and a non-fluorescent quencher.
15

10.   The hairpin primer according to claim 1 containing modified nucleotides, modified internucleotide linkages, or both.

11.   The hairpin primer according to claim 1 wherein said primer
20   contains a terminator nucleotide between the loop sequence and the 5' arm sequence.

12. The hairpin primer according to claim 1 wherein said priming region contains a single nucleotide subject to mutation and said loop sequence is complementary to the portion of said priming region containing said nucleotide.

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13. The hairpin primer according to claim 12 additionally comprising interactive fluorescent label moieties attached to the 3' and 5' arm sequences, whereby incorporation of the primer into a double-stranded nucleic acid detectably alters the fluorescence emitted by said label moieties.

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14. The hairpin primer according to claim 13 wherein said interactive fluorescent label moieties comprise a fluorophore and a non-fluorescent quencher.

15.   In a linear oligonucleotide primer for extension by a DNA
10   polymerase, said primer having a 5' terminus and a 3' terminal region, the improvement comprising adding to said 5' terminus a nucleotide sequence that is complementary to said 3' terminal region to form an hairpin structure comprising a stem and loop, wherein hybridization of the loop to a model oligonucleotide having the same length as the loop and being perfectly complementary thereto does not cause
15   said stem to dissociate.

16.   The improvement according to claim 15 wherein said loop is 5 to 9 nucleotides in length and said stem is 5 to 12 nucleotides in length.

20   17.   The improvement according to claim 15 further comprising adding a terminator nucleotide to the 5' terminus prior to adding said nucleotide sequence that is complementary to said 3' terminal region.

18. In a process for nucleic acid amplification comprising the
25   extension of an oligonucleotide primer by a DNA polymerase, said primer having a 5' terminus and a 3' terminal region, the improvement comprising adding to said 5' terminus a nucleotide sequence that is complementary to said 3' terminal region to form an hairpin structure comprising a stem and loop, wherein hybridization of the

loop to a model oligonucleotide having the same length as the loop and being perfectly complementary thereto does not cause said stem to dissociate.

19.   The improvement according to claim 18 wherein said loop is 5 to 9 nucleotides in length and said stem is 5 to 12 nucleotides in length.

20. A process for nucleic acid amplification comprising the extension by a DNA polymerase of at least one oligonucleotide primer according to claim 1.

21. The process according to claim 20 selected from the group consisting of a polymerase chain reaction (PCR), a strand displacement reaction (SDA), a nucleic acid sequence-based amplification (NASBA), a transcription-mediated amplification (TMA), and a rolling-circle amplification (RCA).

22.   The process according to claim 21 wherein said at least one primer according to claim 1 comprises a pair of amplification primers.

23.   The process according to claim 21 wherein said at least one primer according to claim 1 includes a terminator nucleotide between the loop and the 5' arm.

24. The process according to claim 20 including real-time detection of intended amplification products utilizing separate detector probes having interactive labels, at least one of which is a fluorophore.

25. The process according to claim 20 including real-time detection, wherein said at least one primer additionally comprises interactive fluorescent label

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moieties attached to the 3' and 5' arm sequences, whereby incorporation of the primer
into a double-stranded nucleic acid detectably alters the fluorescence emitted by said
label moieties.

5   26.   The process according to claim 25 wherein said interactive
fluorescent label moieties comprise a fluorophore and a non-fluorescent quencher.

           27. The process according to claim 20 wherein said loop sequence is 5
to 9 nucleotides in length and said complementary 3' and 5' arm sequences form a
10   stem that is 5 to 12 nucleotides in length.

           28. The process according to claim 27 wherein said priming region
contains a single nucleotide subject to mutation and said loop sequence is
complementary to the portion of said priming region containing said nucleotide.

15

           29.   The process according to claim 27 wherein said primer contains
modified nucleotides, modified internucleotide linkages, or both.

           30. The process according to claim 20 wherein said at least one primer
20   according to claim 1 includes a terminator nucleotide between the loop and the 5' arm.

           31. The process according to claim 20 wherein said priming region
contains a single nucleotide subject to mutation and said loop sequence is
complementary to the portion of said priming region containing said nucleotide.

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           32. The process according to claim 31 wherein said primer additionally
comprises interactive fluorescent label moieties attached to the 3' and 5' arm

sequences, whereby incorporation of the primer into a double-stranded nucleic acid
detectably alters the fluorescence emitted by said label moieties.

33. The process according to claim 32 wherein said interactive
fluorescent label moieties comprise a fluorophore and a non-fluorescent quencher.

34. The process according to claim 20 wherein said at one
oligonucleotide primer according to claim 1 comprises a pair of primers.

35. The process according to claim 34 wherein at least one of said
primers according to claim 1 has a loop sequence 5 to 9 nucleotides in length and said
complementary 3' and 5' arm sequences form a stem that is 5 to 12 nucleotides in
length.

36. A kit of reagents for performing amplification of a target nucleic
acid sequence comprising amplification buffer, dNTPs, at least one primer according
to claim 1, and instructions for performing said amplification.

37. The kit of reagents according to claim 36 wherein said at least one
primer according to claim 1 includes a first primer and a second primer differing from
said first primer by a single nucleotide of said loop sequence.

38. The kit of reagents according to claim 37, wherein in each of said
first and second primers the loop sequence is 5 to 9 nucleotides in length and said
complementary 3' and 5' arm sequences form a stem that is 5 to 12 nucleotides in
length.

39. The kit of reagents according to claim 37 wherein said each of said first and second primers includes interactive fluorescent label moieties attached to the 3' end 5' arm sequences, whereby incorporation of each said primer into a double-stranded nucleic acid detectably alters the fluorescence emitted by said label moieties.

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40. The kit of reagents according to claim 36 wherein `in said at least one primer the loop sequence is 5 to 9 nucleotides in length and said complementary 3' and 5' arm sequences form a stem that is 5 to 12 nucleotides in length.

10   41. The kit of reagents according to claim 36 wherein said at least one primer according to claim 1 includes a terminator nucleotide between the loop and the 5' arm.
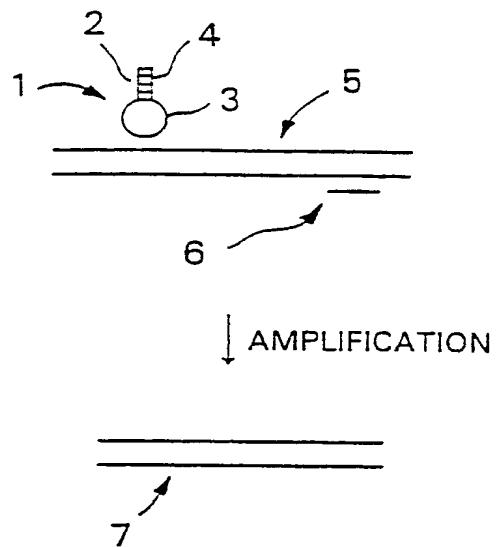
FIG. 1
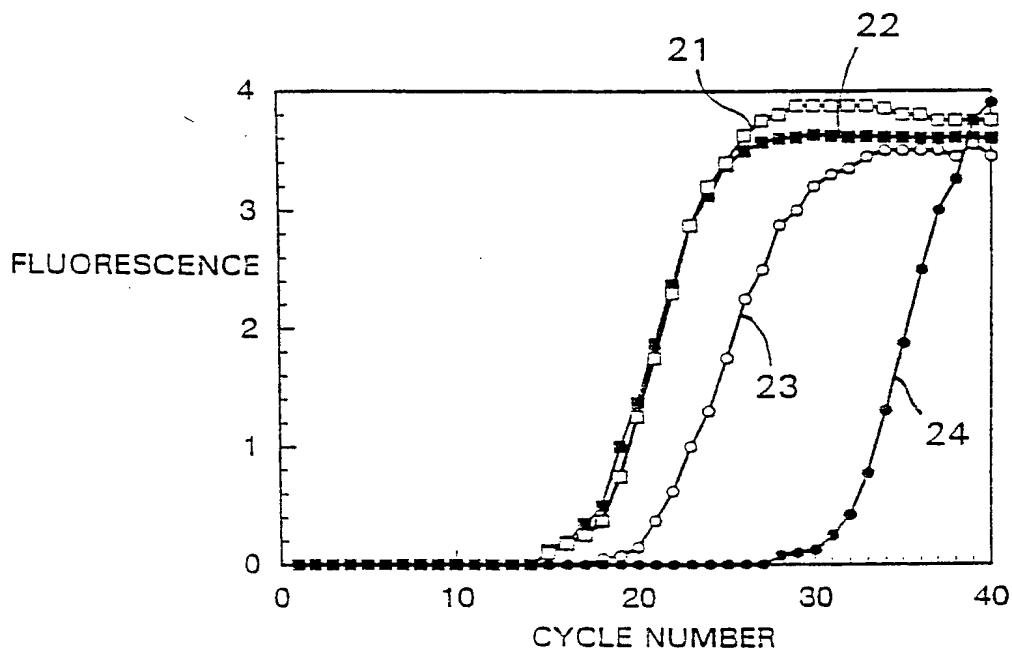
FIG. 2
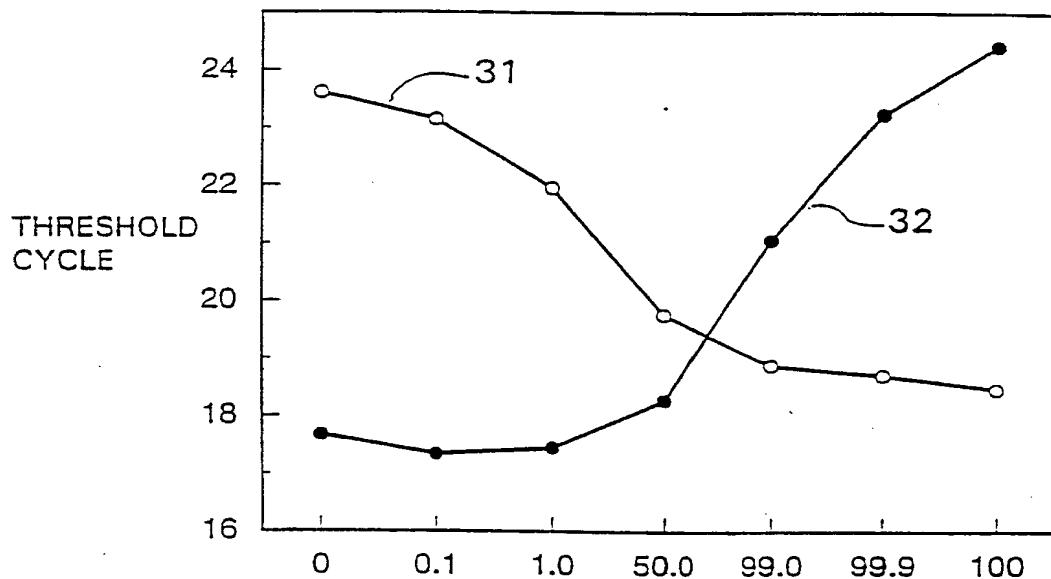
FIG. 3   PROPORTION OF MUTANTS (%)
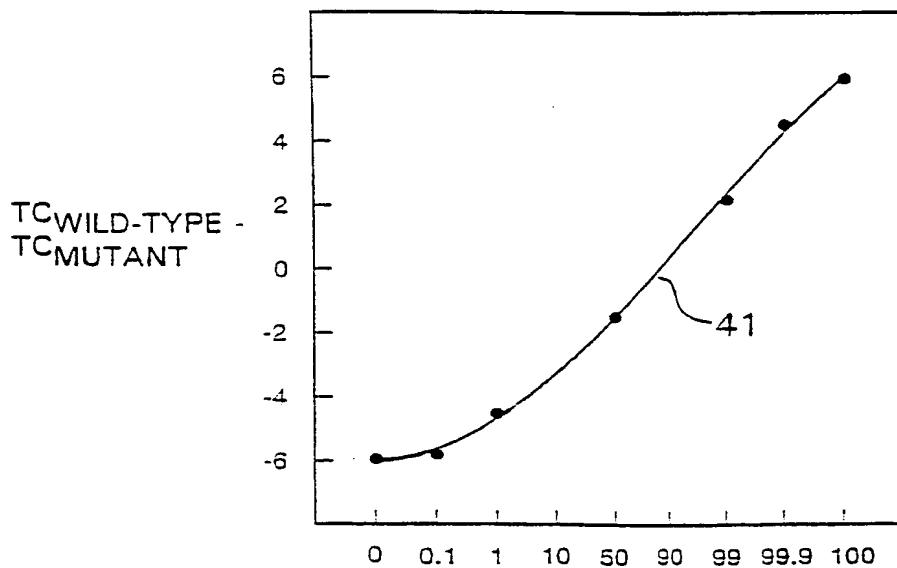
FIG. 4   PROPORTION OF MUTANTS (%)

3/4

```
      G  C
  A  G    C
  G         G
    G     A
      A·T
      A·T
      A·T ──── DABCYL
      G·C
      T·A
      A·T
      G·C
55 ──{
      FI ── 54
```

51
52
53

FIG. 5

FLUORESCENCE
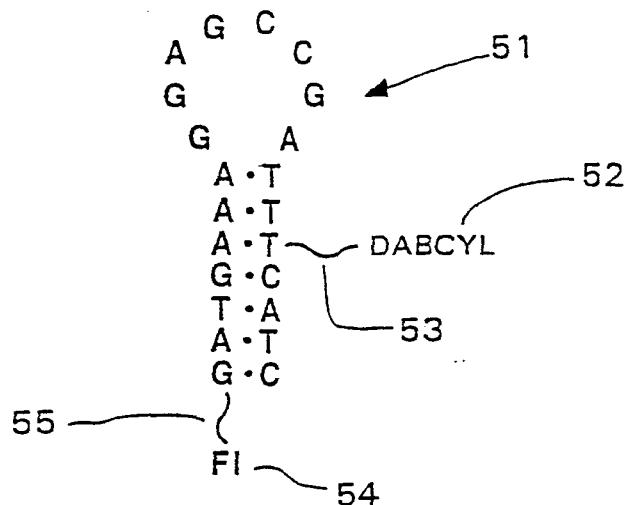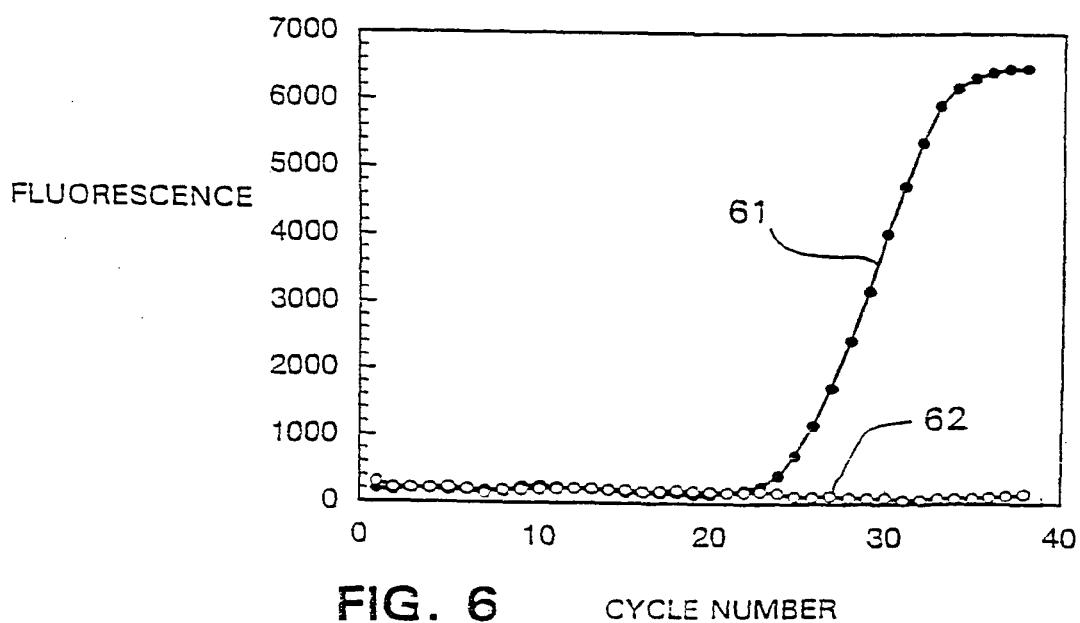
FIG. 6   CYCLE NUMBER

4/4
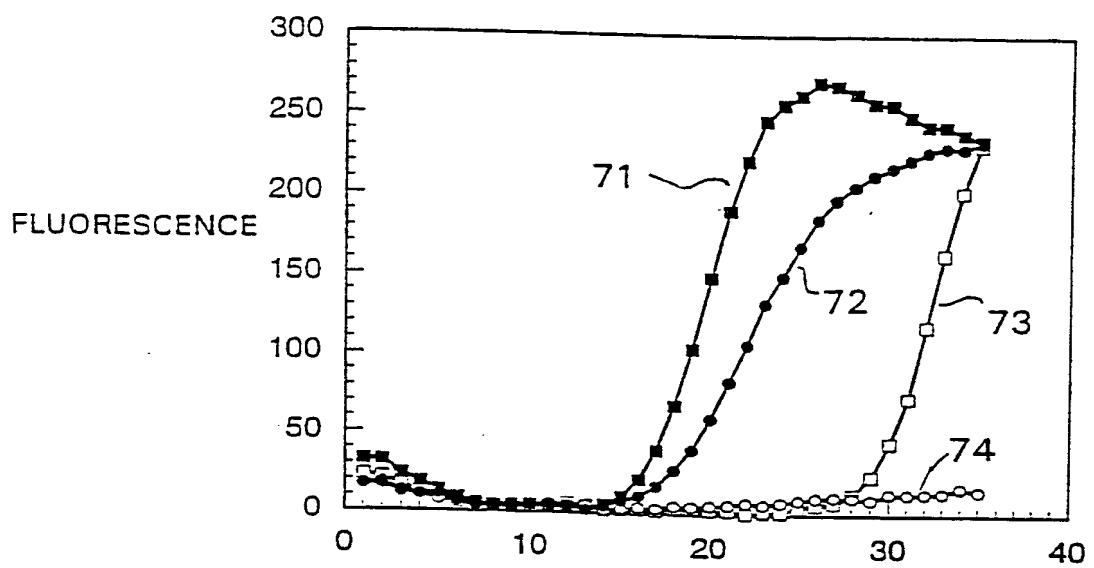
FIG. 7   CYCLE NUMBER

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/11979

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/04; C12P 19/34; G01N 33/53

US CL :536/24.33; 435/91.2, 975

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.3, 24.33, 25.3, 25.4, 25.41; 435/6, 91.1, 91.2, 183, 194, 810; 436/94; 800

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN and WEST

Serach terms: Hairpin, primer, hybridization, PCR, molecular beacon, fluorescence and quencher

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,866,336 A (NAZARENKO et al.) 02 February 1999 (02.02.99), see entire document, especially pages 11, 19-21, and 24.	1-5, 7-10, 15, 16, 18-22, 24-27, 29, 34-36, and 38-40
X	GIESENDORF et al., Molecular Beacons: a new approach for semiautomated mutation analysis. Clin. Chem. March 1998, Vol. 44, No. 3, pages 482-486, especially pages 483.	1-5, 7-10, and 12-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:
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"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 JUNE 2000	0 9 AUG 2000

Name and mailing address of the ISA/US	Authorized officer
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